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Molecular cloning and functional characterization of two forms of Pax8 in the rainbow trout, *Oncorhynchus mykiss*

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Abstract

We have identified two distinct Pax8 (a and b) mRNAs from the thyroid gland of the rainbow trout (*Oncorhynchus mykiss*), which seemed to be generated by alternative splicing. Both Pax8a and Pax8b proteins were predicted to possess the paired domain, octapeptide, and partial homeodomain, while Pax8b lacked the carboxy-terminal portion due to an insertion in the coding region of the mRNA. RT-PCR analysis showed each of Pax8a and Pax8b mRNAs to be abundantly expressed in the thyroid and kidney. *In situ* hybridization histochemistry further detected the expression of Pax8 mRNA in the epithelial cells of the thyroid follicles of the adult trout and in the thyroid primordial cells of the embryo. The functional properties of Pax8a and Pax8b were investigated by dual luciferase assay. The transcriptional regulation by the rat thyroid peroxidase (TPO) promoter was found to be increased by Pax8a, but not by Pax8b. Pax8a further showed synergistic transcriptional activity with rat Nkx2-1 for the human TPO upstream region including the enhancer and promoter. On the other hand, Pax8b decreased the synergistic activity of Pax8a and Nkx2-1. Electrophoretic mobility shift assay additionally indicated that not only Pax8a but also Pax8b can bind to the TPO promoter and enhancer, implying that the inhibitory effect of Pax8b might result from the lack of the functional carboxy-terminal portion. Collectively, the results suggest that for the trout thyroid gland, Pax8a may directly increase TPO gene expression in cooperation with Nkx2-1 while Pax8b may work as a non-activating competitor for the TPO transcription.

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Keywords

Pax8; thyroid gland; Nkx2-1; TTF1; thyroid transcription factor 1; rainbow trout

1. Introduction

The thyroid hormones, thyroxine and triiodothyronine, are produced in all classes of vertebrates, and play crucial roles in the regulation of development, metabolism, homeostasis, and reproduction (Bentley, 1998; Melmed et al., 2011). As to teleost fishes, it is well known that thyroid hormones control flounder metamorphosis (Power et al., 2001) and the parr-smolt transformation of salmonids (Bjornsson et al., 2011). Further, thyroid hormones are shown to affect a wide range of other physiological processes such as gonad maturation in the goldfish (Nelson et al., 2010) and changes in the olfactory system in the salmon during an imprinting period (Lema and Nevitt, 2004), suggesting their widespread importance in fish physiology.

Molecular machinery for thyroid hormone synthesis and secretion operates in the thyroid follicles, which requires the expression of sodium/iodide symporters (NIS), thyroglobulin (TG), thyroid peroxidase (TPO), and thyroid-stimulating hormone (TSH) receptors (Kovacs and Ojeda, 2011). According to the mammalian scenario, iodide is accumulated in the follicular epithelial cells through NIS, and incorporated into tyrosyl residues within TG by TPO. TPO can also catalyze the oxidative coupling of iodotyrosines, followed by storage of the iodinated TG in the colloid. The thyroid hormones are released after endocytosis of the colloid, which is regulated by TSH. This unique phenotype of the thyroid follicular cells is indispensable for thyroid hormone synthesis, and maintained by the fundamental action of a specific set of transcription factors, including Pax8, Nkx2-1 (also known as TTF1, TITF1, or T/EBP), FoxE1 (also called TTF2 or TITF2), HHEX, and TAZ (Damante et al., 2001; De Felice and Di Lauro, 2011; Di Palma et al., 2009; Kimura, 2011).

Pax8 is a member of the Pax family of vertebrate paired-domain proteins, involved in tissue differentiation and organogenesis (Blake et al., 2008; Underhill, 2012). In mammals, it has been documented that Pax8 functions as a key regulator for the maximal transcription of TG (Miccadei et al., 2002), TPO (Miccadei et al., 2002), and NIS genes (Taki et al., 2002). Accordingly, genetic analysis indicated that single or multiple *cis*-acting DNA elements for Pax8 are located in the 5′ upstream region of these genes (Damante et al., 2001; Miccadei et al., 2002; Taki et al., 2002). The Pax8 gene is expressed in the thyroid gland and kidney (Plachov et al., 1990; Zannini et al., 1992). However, Pax8 null mice failed to form only thyroid follicular cells, showing an essential role for Pax8 in the development of these cells (Mansouri et al., 1998; Van Vliet, 2003).

The thyroid glands of most non-mammalian vertebrates are presumed to conserve a similar molecular mechanism for hormone synthesis (Bentley, 1998). Pax8 mRNA was detected in the thyroid primordium and developing thyroid of zebrafish (Wendl et al., 2002), and also in the thyroid gland of *Xenopus laevis* tadpoles (Opitz et al., 2006). It was further reported that in the cultured thyroid glands of *Xenopus* tadpoles, bovine TSH enhanced the expression of Pax8 mRNA (Opitz et al., 2006). To our knowledge, however, there is no experimental

evidence on the functional property of non-mammalian Pax8 in the thyroid gland. In the present study, we have cloned two distinct cDNAs encoding Pax8 isoforms (Pax8a and Pax8b) from the rainbow trout thyroid, and examined their transcriptional activities by dual luciferase assay. Because the rainbow trout has been used as a model animal to study the physiological roles of thyroid hormones in fish (Bres et al., 2006; Suliman and Flamarique, 2013), it is of special significance to elucidate the molecular mechanisms operating in the thyroid gland of this species.

2. Materials and methods

2.1. Animals and sampling

Rainbow trout, *Oncorhynchus mykiss*, of 1.5-years of age were collected at Fuji Trout Hatchery, Shizuoka, Japan, in January and May. Before sampling, the animals were anesthetized with ethyl m-aminobenzoate methanesulfonate (Nacalai tesque, Kyoto, Japan). Various organs, including thyroid tissue with the ventral aorta, were carefully dissected out, immediately frozen in liquid nitrogen, and stored at −80°C in a deep freezer. Rainbow trout embryos from Fuji Trout Hatchery were reared in an aquarium with recirculating fresh water at 14°C, and sampled 15 days after fertilization. In addition, the thyroid and liver were removed from a Wistar rat of 6 weeks (Japan SLC Inc., Shizuoka, Japan) anesthetized with ether. All animal experiments were carried out in compliance with the Guide for Care and Use of Laboratory Animals of Shizuoka University.

2.2. Construction of cDNA library and DNA cloning

Total RNA was extracted from 85 ventral aortae with thyroid follicles using TRIZOL reagent (Invitrogen Corp., Carlsbad, CA, USA), and poly(A)$^+$ RNA was prepared with oligo-dT coated latex beads (Oligotex-dT30 super; Takara, Kyoto, Japan), according to the manufacturer's instructions. cDNA was prepared with a ZAP Express cDNA synthesis kit (Agilent Technologies, La Jolla, CA, USA), and a cDNA library was constructed using a ZAP Express cDNA Gigapack III Gold cloning kit (Agilent Technologies). The resultant cDNA library contained 2.0×10^7 recombinant rhambda phages, and was amplified to 1.0×10^9 plaque-forming units (pfu)/ml.

2.3. Preparation of cDNA probe

Poly(A)$^+$ RNA (0.5 μg) from the rat thyroid gland was reverse-transcribed in 20 μl buffer containing 80 pmol of dT-adaptor primer (5′-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT), 1 mM dNTP, 20 units RNase inhibitor (Toyobo, Osaka, Japan), and 10 units Rous-associated virus 2 (RAV-2) reverse transcriptase (Takara), as described previously (Suzuki *et al*. 1992). Polymerase chain reaction (PCR) was subsequently carried out with rat Pax8 sense primer (5′-TACTACGAGACTGGCAGCAT) and rat Pax8 antisense primer (5′-GTTGAATGGCTGCTGCACTT) using a Program Temp Control System, PC-701 (Astec, Fukuoka, Japan). The amplification profile for 30 cycles was: dissociation at 94 °C for 1.5 min, annealing at 55 °C for 1.5 min, and extension at 72 °C for 2.5 min. The final cycle included polymerization for 8 min. Amplification products were separated by electrophoresis, and a major band was subcloned into pGEM-3Z vector (Promega, Madison,

WI, USA). Sequencing reactions were conducted with a thermo sequenase cycle sequencing kit (USB, Cleveland, OH, USA), and nucleotide sequences were analysed using a Li-Cor automated DNA sequencer model 4200L-2G (Li-Cor, Lincoln, NE, USA). A DNA probe was synthesized from the cloned rat Pax8 cDNA fragment, using a digoxigenin (DIG)-high prime kit (Roche Diagnostics GmbH, Mannheim, Germany).

2.4. DNA cloning and sequence analysis

Approximately 80,000 recombinants from the amplified cDNA library were screened by plaque hybridization. Hybridization with the above cDNA probe and post-hybridization washing were performed, basically according to the manufacturer's instructions. Hybridization signals were detected with 25mM CSPD chemiluminescent 1, 2-dioxetane enzyme substrate (Tropix, Bedford, MA, USA), on Hyperfilm-ECL film (GE Healthcare, Buckinghamshire, UK) after incubation with alkaline phosphatase-conjugated anti-DIG Fab antibody (Roche). The pBK-CMV phagemid vectors with inserts were excised *in vivo* from the ZAP express vectors of positive recombinants, using the ExAssist helper phage (Agilent Technologies). The nucleotide sequences of these DNAs were analysed using a Li-Cor automated DNA sequencer. The sequence data were analyzed using Genetyx, ver. 8 (Genetyx Corporation, Tokyo, Japan)

2.5. Phylogenetic analysis

The amino acid sequences of Pax2/5/8 proteins from the rainbow trout, zebrafish, *Xenopus laevis*, chicken, mouse, and human were aligned using Clustal W (Clustal W, 2013; Thompson et al., 1994) after alignment parameters were set according to an instruction manual by Hall (Hall, 2011). An optimal unrooted tree was inferred by the NJ method (Saitou and Nei, 1987) in the MEGA program ver. 5.2 (Tamura et al., 2011). The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000), and confidence in the NJ tree was assessed with 10,000 bootstrap replications (Felsenstein, 1985).

2.6. Tissue distribution of Pax8 mRNAs

Total RNA was extracted from various tissues of rainbow trout, using TRIZOL reagent (Roche). After treatment with deoxyribonuclease I (Takara), 10 μg total RNA was subjected to RT-PCR, as described above. The following pairs of specific primers were synthesized and used for detection of each of Pax8a and Pax8b mRNAs: 5′-GAGCAGACTGTGTACCCTCT and 5′-CAGCGATTGCAGCTCCAGAA for the Pax8a; and 5′-TACGATGGATGCTGCCCTTCT and 5′-AATCAGATTGATGACAACCG for the Pax8b. Elongation factor 1-α (EF1-α) cDNA (Accession number BE669055) was also amplified, as an endogenous control, with specific primers: 5′-GGCTGGTTCAAGGGATGGAA and 5′-ATTGGAGGGTCGTTCTTGCT. An aliquot of 5 μl of each amplified product was electrophoresed through an ethydium bromide-stained 2% agarose gel, and photographed with a FAS-III digital camera system (Nippon Genetics, Tokyo, Japan).

2.7. Localization of Pax8 mRNA

A cDNA fragment of 464 bp was first amplified from the conserved region of rainbow trout Pax8a and Pax8b cDNAs by PCR using Pax8 cRNA primers (5′-AACAGGAAATGTCCCCAGAG and 5′-GAGATGGTCGTAGGCTGCAG), and subcloned into pGEM-3Z vector (Promega), basically as described above. Antisense and sense cRNA probes were then prepared from this subcloned fragment by *in vitro* transcription, using a DIG RNA labelling kit (Roche). *In situ* hybridization histochemistry was carried out on paraffin sections of the thyroid gland, basically as described before (Suzuki et al., 1997). Briefly, tissue sections (4 μm) of the thyroid were digested with 5 μg/ml proteinase K at 37 °C for 20 min, and fixed in 4% formaldehyde at 4°C for 20 min. After incubation at 65°C overnight with the hybridization buffer, the sections were washed in 2× SSC/50% formamide at 58°C for 30 min, incubated in 10 μg/ml RNase A solution at 37°C for 30 min, and washed once in 2× SSC and twice in 0.2× SSC at 50°C for 20 min each time. The sections were then incubated in a 1:500 diluted solution of anti-DIG antibody, and stained with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP). Whole-mount *in situ* hybridization histochemistry (WISH) was further performed with the same cRNA probes, basically as described previously (Hidaka et al., 2004). After WISH, some specimens were embedded in paraplast wax, and 6 μm sections were cut for observation at the cellular level.

2.8. Reporter constructs and expression vectors

Genomic DNA was prepared from the rat liver by phenol/chloroform extraction. The 5′-upstream region of Wistar rat TPO gene (AB830619) was amplified from the genomic DNA by PCR using TPO5 primers (5′-ACCTCTCTGGCTCCTTCAAT and 5′-CCACTGAAGAAGCAGGCTGT), basically as described above. The amplified fragment was then digested with *Eco*R V and *Apa*L I, and blunt-ended with a DNA blunting kit (Takara). The resultant 5′-flanking region (−713/+62) was inserted to the pGL3-basic firefly luciferase reporter vector (Promega). The human TPO 5′-upstream region (about −6300/+14)/promoterless luciferase vector pSVOAL-AΔ5′ was as previously described (Kikkawa et al., 1990; Mizuno et al., 1991). The trout Pax8a cDNA/pBK-CMV and trout Pax8b cDNA/pBK-CMV were prepared by *in vivo* excision, as described above. The rat Nkx2-1 cDNA (AB22130)/pBK-CMV was prepared as previously reported (Suzuki et al., 2007). The lac promoter was deleted from the pBK-CMV plasmids for maximal eukaryotic expression.

2.9. Transfection and reporter assays

Transfection of the HeLa cell line was carried out with LipofectAMINE 2000 reagent (Invitrogen), following the manufacturer's instructions. Approximately 2×10^4 cells were seeded onto 96-well plates and allowed to adhere overnight. Cells were cotransfected with 280 ng of pGL3-basic firefly luciferase reporter vector (Promega) including the rat TPO promoter or pSVOAL-AΔ5′ luciferase vector containing the human TPO 5′-upstream region, 28 ng of synthetic *Renilla* luciferase reference vector containing the herpes simplex virus thymidine kinase promoter (phRL-TK; Promega), and 5 ng of pBK-CMV, trout Pax8a/pBK-CMV, trout Pax8b/pBK-CMV, and/or rat Nkx2-1/pBK-CMV. Transfected cells were

lysed with the Dual-Glo luciferase reagent (Promega) 48 h post-transfection, and luciferase light outputs were measured with a luminometer, Luminescencer JNR AB2100 (ATTO, Tokyo, Japan). In each well, the ratio of firefly luciferase to *Renilla* luciferase activity was calculated after normalization to the transfection reference vector. Results were presented as the mean ± SEM.

2.10. Electrophoretic mobility shift assay (EMSA)

The coding regions of trout Pax8a and Pax8b cDNAs were obtained from their respective cloned cDNAs by PCR with the following primers: 5′-AGAGAGAATTCGATATGTCCAACAACACCGGA and 5′-TGTGTGTCGACTGGGAGCTAGAGATGGTCGTA for the Pax8a; and 5′-AGAGAGAATTCGATATGTCCAACAACACCGGA and 5′-TGTGTGTCGACTCATCTTTAAGCTCTGATATT for the Pax8b. The amplified fragments were digested with *Eco*R I and *Sal* I, and ligated in-frame into the *Eco*R I/*Sal* I site of pET32a (Merck, Damstat, Germany). Each of the trout Pax8a/pET32a and trout Pax8b/pET32a was introduced into *E. coli* BL21 (DE3) codon plus RP (Agilent Technologies). His-Tag-fused recombinant Pax8 proteins were then produced by induction with Overnight Express Autoinduction system 1 (Merck) and purified with His-Tag Mag beads (Toyobo). Two types of synthetic double-stranded oligonucleotides were used and labelled with 6-FAM fluorescein as probes: *i.e.* 5′-[6-FAM]CTGTCTAAGCTTGAGTGGGCATCA (oligonucleotide CT) (Zannini et al., 1992), and 5′-[6-FAM]GAACCAGGGATTCTTCACACTTCATAGAGCAGCT (oligo B) (Kikkawa et al., 1990). The binding reaction was carried out in a 10 μl mixture including 250 ng of the recombinant Pax8a or Pax8b, 100 fmol of the FAM-labelled probe, 200 ng of poly (dI-dC), 10 mM HEPES (pH 7.9), 0.4 mM DTT, 50 mM NaCl, and 4% glycerol, at 37 °C for 30 min. In some assay, 10 pmol of the non-labelled oligonucleotide was added to the reaction mixture to assess the specific binding. The samples were then subjected to electrophoresis on a 6% polyacrylamide gel. Fluorescence signals were captured with the Molecular Imager PharosFX system (Bio-Rad, Hercules, CA, USA).

3. Results

3.1. Molecular cloning of rainbow trout Pax8 cDNAs

In order to clone rainbow trout Pax8 cDNAs, at first a partial fragment of rat Pax8 cDNA was amplified from rat thyroid cDNAs by PCR using rat Pax8 sense and antisense primers. After gel electrophoresis, a distinctive cDNA band of approximately 250 bp was isolated and subjected to sequence analysis, confirming that its nucleotide sequence corresponded to rat Pax8 cDNA. This cDNA fragment was then used as a probe to screen 80,000 recombinants from the rainbow trout cDNA library. Sequence analysis revealed the complete nucleotide sequences of two distinct cDNAs encoding Pax8 isoforms, which we have designated as Pax8a and Pax8b (Fig. 1).

3.2. Rainbow trout Pax8 cDNAs and proteins

The cDNAs for rainbow trout Pax8a (accession number AB828387) and Pax8b (AB828388) were composed of 2,734 and 3,290 bases, respectively, excluding the poly(A) tail. The

Pax8a protein consisted of 458 amino acid residues and contained three characteristic domains: *i.e.* the paired domain, octapeptide, and partial homeodomain (Figs. 1 and 2). The nucleotide sequence of Pax8b cDNA was identical with that of Pax8a cDNA except for three regions. The Pax8b cDNA lacked the 5′ non-coding terminus of 30 bases seen in Pax8a cDNA (region I) (data not shown) and a coding sequence corresponding to the position 23 to 46 of Pax8a cDNA (region II), while an addition of a 610-nucleotide sequence was found at the position 860 of Pax8b cDNA (region III) (Fig. 1). Because of a deletion of region II, Pax8b was predicted to lack an N-terminal portion of the paired domain (Figs. 1 and 2). Additionally, an insertion of region III changed the reading frame of Pax8b cDNA, which resulted in the deletion of the C-terminal portion, corresponding to that from Asp294 to Leu458 in Pax8a (Figs. 1 and 2). The site of this insertion corresponded to the boundary between exon 8 and exon 9 in human Pax8 gene (Poleev et al., 1995) (Fig. 2). The trout Pax8a showed 67.7%, 67.1%, 67.2%, and 76.0% amino acid similarity to human Pax8 (ENST00000429538), mouse Pax8 (ENSMUST00000028355), *Xenopus* Pax8 (AF179301), and zebrafish Pax8 (ENSDART00000029014), respectively. In vertebrates, *Pax* genes are classified into four subgroups comprising *Pax1*/*9*, *Pax2*/*5*/*8*, *Pax3*/*7*, and *Pax4/6* (Underhill, 2012). To verify the phylogenetic position of trout Pax8s, an NJ tree was constructed using the amino acid sequences of vertebrate Pax2/5/8 proteins (Fig. 3). The topology of this tree indicated that both trout Pax8a and Pax8b are assigned to the cluster containing zebrafish, *Xenopus*, mouse, and human Pax8 proteins.

3.3. Tissue distribution and histological localization of Pax8 mRNAs

Tissue distribution of the trout Pax8a and Pax8b mRNAs was examined by RT-PCR using specific primers. The RT-PCR analysis showed that both the Pax8a and Pax8b mRNAs were expressed mainly in the thyroid tissue and kidney (Fig. 4). The Pax8a mRNA was also observed very weakly in the ovary and testis (Fig. 4). Because the thyroid tissue used in the RT-PCR analysis included the ventral aorta, the expression site of trout Pax8 mRNA was examined by *in situ* hybridization histochemistry. Specific antisense probes that distinguish Pax8a mRNA from Pax8b mRNA could not be made due to the very high nucleotide identity between these molecules. Therefore, we utilized a cRNA probe that can bind to both the Pax8a and Pax8b mRNAs. The hybridization of paraffin sections with this probe exhibited that the signals for Pax8 mRNA resided in the monolayered epithelial cells of thyroidal follicles in the adult trout (Fig. 5A). Whole mount *in situ* hybridization histochemistry was also conducted for the trout embryos 15 days postfertilization. It is known that in the zebrafish, the thyroid anlage appears in a ventral region of the midline endoderm near the heart tube (Porazzi et al., 2009; Wendl et al., 2007). Accordingly, hybridization signals for Pax8 transcripts were detected, as a single patch, at the posterior end of the ventromedial line in the pharyngeal region (Fig. 5C). Paraffin sectioning of the whole mounts revealed that the signals were confined to the thyroid primordium close to the developing ventral aorta (Fig. 5D).

3.4. Functional divergence of rainbow trout Pax8s

Transient transfection studies were carried out to elucidate the transcriptional activities of rainbow trout Pax8a and Pax8b. Initially, HeLa cells were transfected with the −713 bp rat TPO promoter-luciferase construct and with either the trout Pax8a or Pax8b-expression

vectors (Fig. 6A). The luciferase activities indicated that Pax8a stimulated promoter transcription by approximately 4-fold as compared with the control vector, whereas transcription was not induced by Pax8b (Fig. 6A). In mammals, it is reported that Pax8 and Nkx2-1 could cooperatively activate the TPO gene via interplay of the enhancer and gene promoter (Miccadei et al., 2002). Therefore, we next used the 6.3-kbp human genomic fragment, which includes the TPO promoter and enhancer, to assess the effects of Pax8a and/or Pax8b on the transcriptional property of Nkx2-1. Transfection with rat Nkx2-1 showed an about 4-fold increase in luciferase activity from the 6.3-kbp TPO upstream region (Fig. 6B), agreeing well with the result by Miccadei et al. (Miccadei et al., 2002). Pax8a or Pax8b alone did not increase luciferase activity, but co-transfection with Pax8a and rat Nkx2-1 induced a synergistic increase in luciferase transcription by nearly 25-fold (Fig. 6B). In contrast, Pax8b did not activate reporter transcription even when co-transfected with Nkx2-1, and rather reduced the synergistic increase in reporter activity by Pax8a and Nkx2-1 (Fig. 6B).

3.5. Binding specificity of rainbow trout Pax8s

Binding sites of trout Pax8a and Pax8b were investigated by EMSA using two types of synthetic oligonucleotides: *i.e.* oligonucleotide CT (Zannini et al., 1992) (Fig. 7A), and oligo B (Kikkawa et al., 1990) (Fig. 7B). The former was derived from the region −64 to −41 in the rat TPO promoter, which includes a binding site for mammalian Pax8 (Francis-Lang et al., 1992; Zannini et al., 1992). The latter corresponds to a 34-bp portion in the enhancer region located approximately 5.5 kbp upstream of the human TPO gene, which binds to mammalian thyroid-specific proteins (Kikkawa et al., 1990). The EMSA using the FAM-labelled oligonucleotide probes demonstrated distinctive retarded bands representing the interaction of Pax8a with oligonucleotide CT (Fig. 7A, lane 1) and oligo B (Fig. 7B, lane 1). These retarded bands were eliminated by addition of non-labelled oligonucleotide CT (Fig. 7A, lane 2) or oligo B (Fig. 7B, lane 2), confirming the specific binding of Pax8a and these oligonucleotides. Similarly, shifted electrophoretic bands were detected when Pax8b was tested for oligonucleotide CT (Fig. 7A, lane 3) and oligo B (Fig. 7B, lane 3). Those shifted bands were also abrogated by addition of non-labelled oligonucleotide CT (Fig. 7A, lane 4) or oligo B (Fig. 7B, lane 4). No retarded complexes were observed when the Tag polypeptide, produced from the pET32a, was mixed with oligonucleotide CT (Fig. 7A, lane 5) or oligo B (Fig. 7B, lane 7). In addition, it was confirmed that rat Nkx2-1 can specifically bind to the oligo B (Fig. 7B, lanes 5 and 6), as shown previously (Mizuno et al., 1991).

4. Discussion

In the present study, we identified two distinct cDNAs encoding Pax8a and Pax8b from the thyroid gland of rainbow trout. The deduced amino acid sequence showed that three characteristic domains, *i.e.* the paired domain, octapeptide, and short homeodomain, are highly conserved in trout Pax8a. On the other hand, trout Pax8b was predicted to be a smaller protein, but the topology of the phylogenetic tree demonstrated that this protein also belongs to the Pax8 cluster. Because the nucleotide sequence of Pax8b cDNA was identical with that of Pax8a cDNA except for two deleted portions and one inserted portion, it seems

likely that Pax8b mRNA is produced by alternative splicing from the same gene as Pax8a mRNA.

In vertebrates, Pax transcripts from the Pax2/5/8 subgroup tend to be extensively alternatively spliced (Short et al., 2012). For *Xenopus laevis*, Pax8 gene consists of 12 exons, and gives rise to two forms of transcripts, *i.e.* a bona fide transcript from all the exons and a spliced transcript skipping exon 9, in the developing embryos (Short et al., 2012). The human PAX8 gene is also composed of 12 exons, and cDNA cloning and RT-PCR analysis identified at least five different alternatively spliced transcripts encoding human PAX8 isoforms (Pax8a-e) (Poleev et al., 1995). Among these transcripts, three splice forms encoding PAX8a, PAX8b, and PAX8c were shown to be expressed in the thyroid gland. Nothing is, however, known about whether alternative splicing events occur during Pax8 gene expression in the thyroid of the other vertebrates. For *X. laevis*, the above Pax8 splice isoform was identified by RT-PCR using RNA from whole embryos as a template (Short et al., 2012), but whole mount in situ hybridization of embryos localized Pax8 mRNA to the hindbrain, spinal cord, optic vesicle, and pronephric kidney (Heller and Brandli, 1999). In addition, Pax8 and Pax2a (Pax2.1) were shown to be expressed in the thyroid of zebrafish (Fagman and Nilsson, 2010), although there was no report on the alternatively spliced isoforms of these molecules in the fish thyroid. Therefore, the present study provides first evidence to suggest the presence of alternatively spliced Pax8 transcripts in the non-mammalian thyroid gland.

Functional analysis by dual luciferase assay revealed that like mammalian bona fide Pax8 (Miccadei et al., 2002), trout Pax8a can enhance reporter transcription from the rat TPO promoter and further induce a synergistic increase in transcription from the human TPO upstream region together with Nkx2-1. In contrast, trout Pax8b did not show these transcriptional activities, and rather inhibited the synergistic activity of Pax8a and Nkx2-1. To our knowledge, this is also the first finding to show the inhibitory effect of the alternatively spliced Pax8 isoform. The unique properties of Pax8b could result from the one deletion and/or one insertion seen in the coding region of Pax8b cDNA. Because of the deletion, Pax8b was predicted to lack an N-terminal portion of the paired domain that makes sequence-specific contacts with DNA (Underhill, 2012). Generally, a β-hairpin structure is formed in the N-terminal portion of the paired domain, and assists DNA binding through contact to the sugar backbone (Wheat et al., 1999). A clinical and genetic research on the probands suffering from congenital hypothyroidism identified a heterozygous *PAX8* mutation, causing a Leu16Pro (Fig. 2) substitution in the β-hairpin portion, and further showed the mutated PAX8 to have defects in the binding to oligonucleotide CT (Narumi et al., 2012). By contrast, although trout Pax8b lacked Leu at the corresponding position, our EMSA indicated that Pax8b could specifically bind to the same oligonucleotide CT, as well as oligo B, possibly because of a ‘rescue’ effect made by a simultaneous deletion of 7 amino acid residues flanking the Leu. Therefore, the deletion in Pax8b does not seem to make a significant contribution to the transcriptional inability of this factor.

On the other hand, the insertion in Pax8b cDNA resulted in a reading-frame shift, by which Pax8b lost a C-terminal homologous portion of 155 amino acid residues seen in Pax8a. For mammalian Pax proteins, the C-terminal portion is known to be involved in protein/protein

interaction (Thompson and Ziman, 2011). Molecular dissection analysis indicated the presence of a transactivation domain and an inhibitory domain in the C-terminal portion of mouse Pax8 (Dorfler and Busslinger, 1996) (Fig. 2). Human Pax8c, lacking these domains together, showed a drastic reduction in the transcriptional activity from a TG promoter, as compared with human full-length Pax8a (Poleev et al., 1995). It was further reported that a mammalian Pax8 isoform, which lacks a longer C-terminal portion including both transactivation and inhibitory domains, induced transcription from the rat TG promoter in corporation with Nkx2-1, but the transcriptional activity was substantially low as compared with that by synergy of full-length Pax8 and Nkx2-1 (Di Palma et al., 2003). For trout Pax8, both the transactivation and inhibitory domains were highly conserved in the C-terminal portion of Pax8a, whilst these domains were lost from Pax8b. Taken together, these lines of evidence suggest that the loss of the C-terminal homologous portion may be the main cause of the transcriptional inability and inhibitory effect of Pax8b. It is noteworthy that the insertion site in trout Pax8b cDNA corresponded to the 3′-terminus of exon 8 in human Pax8 gene, after which a reading-frame shift occurs in human Pax8c mRNA (Poleev et al., 1995). This finding implies that the molecular mechanisms underlying the alternative splicing to generate an apparently defective Pax8 isoform in the thyroid may be conserved from fish to mammals, thereby giving rise to this isoform in many fish, and further that this Pax8 isoform may have an essential role in the thyroid follicular cells, together with intact Pax8.

The present study suggests that in the trout thyroid *in vivo*, Pax8a is involved in up-regulating the expression of TPO gene, whereas Pax8b acts as a non-activating competitor that can reduce the effects of Pax8a. However, the actual expression of TPO gene could be regulated through more complex but coordinated interactions of transcription factors and other intranuclear factors. For instance, FoxE1 gene is present in fish genomes together with Pax8 gene (Ensembl, 2013), and the FoxE1 transcripts are observed in the thyroid of zebrafish during development (Nakada et al., 2009). In mammals, FoxE1 is shown to inhibit the transcriptional activity of Pax8 on the TPO gene and further that of Nkx2-1 on the TG gene, as a selective repressor (Perrone et al., 2000). Nevertheless, it was reported that Pax8 was indispensable for the FoxE1 gene expression in the mouse thyroid primordium (Parlato et al., 2004), and that the FoxE1 mRNA levels were positively regulated by Pax8 in the FRTL-5 thyroid cells (Di Palma et al., 2011). Given these data, it is possible that with its direct activating effect, Pax8a might be also engaged in down-regulating the TPO gene indirectly by facilitating the FoxE1 gene expression. In this case, Pax8b might exhibit opposite functions on the TPO gene expression with a direct inhibitory effect and an indirect activating effect through decreased levels of FoxE1. With these opposing actions, Pax8a and Pax8b might contribute to the fine control of the TPO gene expression *in vivo*.

In this study, we identified two forms of trout Pax8 mRNAs which could arise from the same gene. Possibly due to a whole-genome duplication or a series of local duplications, ray-finned fish (Actinopterygii) contain more copies of many genes than other vertebrates (Ravi and Venkatesh, 2008). It is further proposed that salmonids have undergone additional tetraploidization, on the basis of the comparison of the chromosome number and amount of DNA per cell nucleus in fish (Danzmann et al., 2008; Ohno et al., 1968). Hence, there is a

possibility that other Pax8 genes might exist in the trout genome and be expressed in the thyroid gland. In the zebrafish, Pax2a mRNA, as well as Pax8 mRNA, is found in the developing thyroid, and shown to be indispensable for the proper thyroid development (Wendl et al., 2002). Although we did not succeed in cloning Pax2 cDNA, Pax2 might be also expressed in the trout thyroid. Furthermore, the Pax2 gene and other Pax8 genes could generate multiple splice variants. Collectively, in the rainbow trout, thyroid-characteristic genes might be controlled via a more complicated molecular network than in mammals.

5. Conclusions

In the present study, we have identified alternatively spliced mRNAs encoding Pax8a and Pax8b in the rainbow trout thyroid gland. This is the first report to indicate the presence of alternatively spliced Pax8 transcripts in the non-mammalian thyroid gland. Moreover, we have first examined the transcriptional activities of non-mammalian Pax8 for the thyroid-specific gene, and shown the functional difference between Pax8a and Pax8b: *i.e.* Pax8a directly transactivated TPO gene expression in cooperation with Nkx2-1 while Pax8b functioned as a non-activating competitor for the TPO transcription. These findings are important for understanding the complex but coordinated gene regulatory networks of transcription factors in the fish thyroid.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. We first identified alternatively spliced Pax8 mRNAs in the non-mammalian thyroid.
2. These mRNAs, encoding Pax8a and Pax8b, were found in the rainbow trout thyroid.
3. Pax8a directly transactivated TPO gene expression in cooperation with NKX2-1.
4. Pax8b worked as a non-activating competitor for the TPO transcription.
5. The results are important for understanding gene regulation in the fish thyroid.

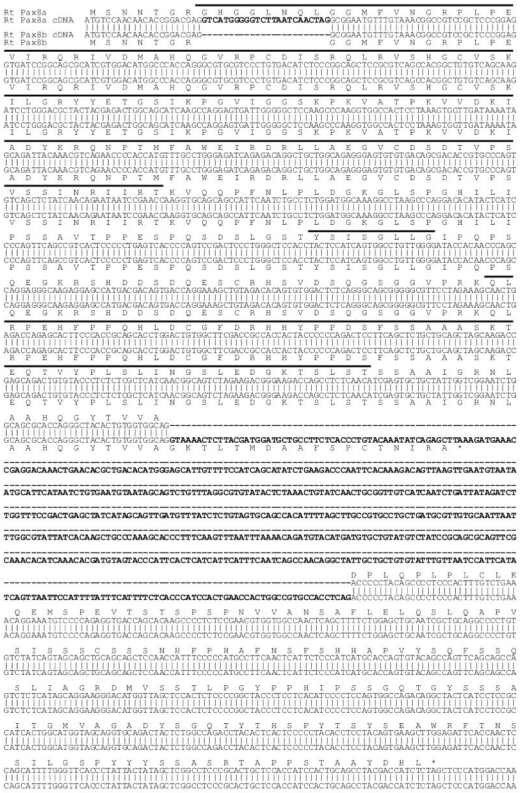


Figure 1.

Nucleotide sequences encompassing the coding regions of Pax8a and Pax8b cDNAs from the rainbow trout (AB828387, AB828388), and their deduced amino acid sequences. Thick lines above the Pax8a amino acid sequence indicates, from N- to C-terminus, the predicted paired domain, octapeptide, and truncated homeodomain. Gaps marked by hyphens (−) are inserted to optimize homology. Vertical lines represent the nucleotide identity between the Pax8a and Pax8b cDNAs, and bold fonts highlight the nucleotides in two inserted/deleted regions. Nucleotides are numbered from 5′ to 3′, beginning with the first base in the coding region. The termination codons are denoted with the asterisk. Rt: Rainbow trout.

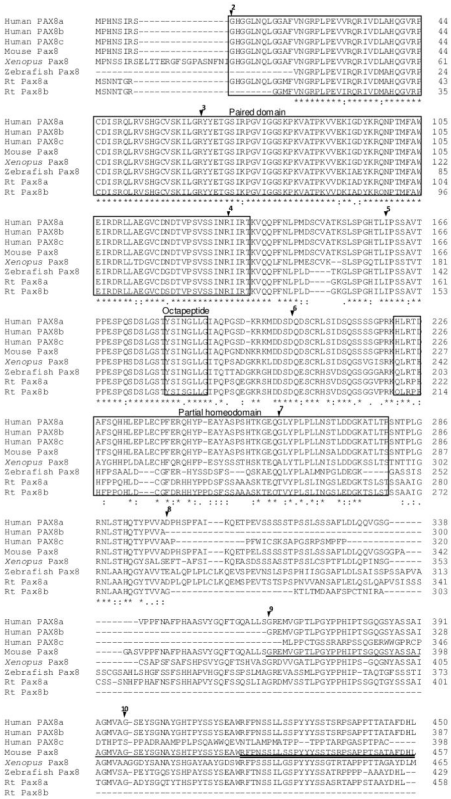


Figure 2.

Comparison of amino acid sequences of Pax8 proteins from the rainbow trout (AB828387, AB828388), zebrafish (ENSDART00000029014), *Xenopus laevis* (AF179301), mouse (ENSMUST00000028355), and human (ENST00000429538; (Poleev et al., 1995). These sequences are aligned using Clustal W (Thompson et al., 1994). Gaps marked by hyphens (−) are inserted to optimize homology. Asterisks denote identical amino acid residues, while colons and periods indicate amino acid residues of strongly similar properties, scoring > 0.5 in the Gonnet PAM 250 matrix, and of weakly similar properties, scoring =< 0.5, respectively. The trout Pax8a and Pax8b share three highly conserved domains with Pax8 from other species, depicted by boxes: *i.e.* the paired domain, octapeptide, and partial homeodomain. The positions of introns in the human Pax8 gene are indicated by numbered arrowheads (Poleev et al., 1995). The transactivation region and adjacent inhibitory domain of mouse Pax8 are indicated, respectively, by single and double underlining (Dorfler and Busslinger, 1996). Rt: Rainbow trout.

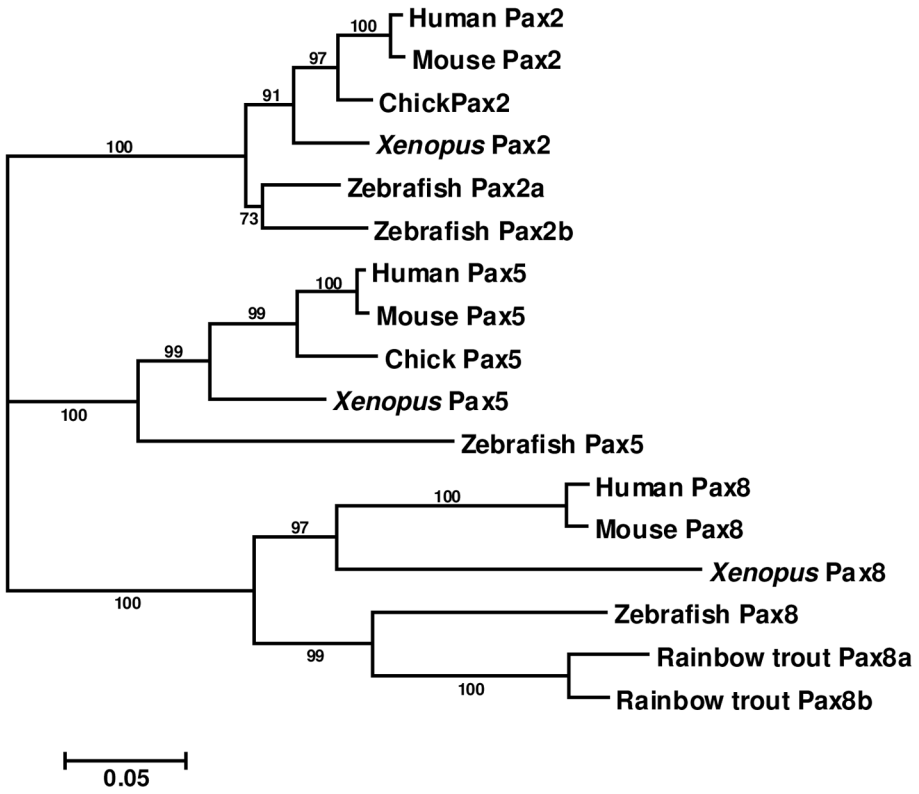


Figure 3.

Phylogenetic tree of the Pax2/5/8 proteins from the rainbow trout (AB828387, AB828388), zebrafish (BC066690, BC162214, ENSDART00000076986, ENSDART00000029014), *Xenopus laevis* (AF179300, NM001085768, AF179301), chicken (AB026496, AB004249), mouse (ENSMUST00000174490, ENSMUST00000014174, ENSMUST00000028355), and human (ENST00000428433, ENST00000358127, ENST00000429538), obtained by the neighbour joining method (Saitou and Nei, 1987). The length of each branch is shown in the units of the number of amino acid differences per residue, and the numbers around the interior branches are bootstrap probabilities (percent; 10,000 replicates). The topology has confirmed that trout Pax8a and Pax8b belong to the cluster containing zebrafish, *Xenopus*, mouse, and human Pax8 proteins.

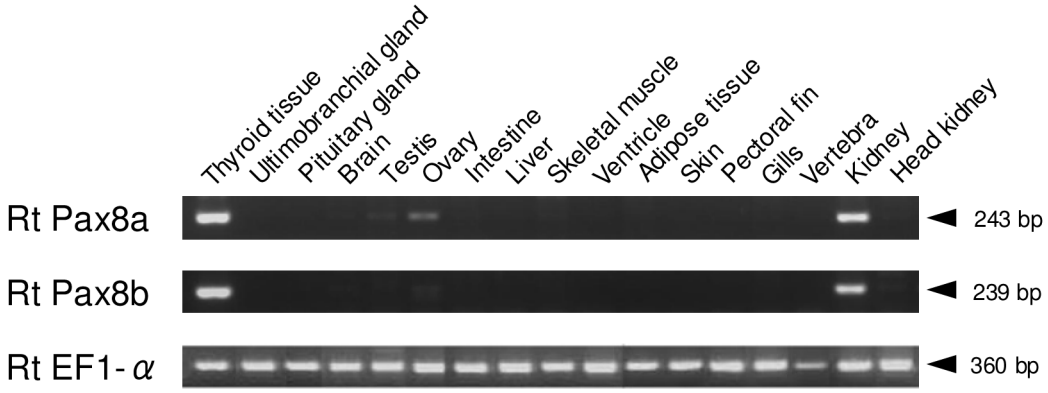


Figure 4.

Tissue distribution of Pax8 mRNAs in the rainbow trout, determined by RT-PCR analysis. Total RNA (10 μg) from various trout tissues were reverse-transcribed and amplified by PCR with specific Pax8 primers. A distinctive band for Pax8a mRNA was detected in the thyroid tissue, including the ventral aorta, and kidney, and faintly in the testis and ovary. The Pax8b mRNA was expressed in the thyroid tissue and kidney. The expression of EF1-α mRNA, an endogenous control, was seen in all the tissues examined. Rt: Rainbow trout.

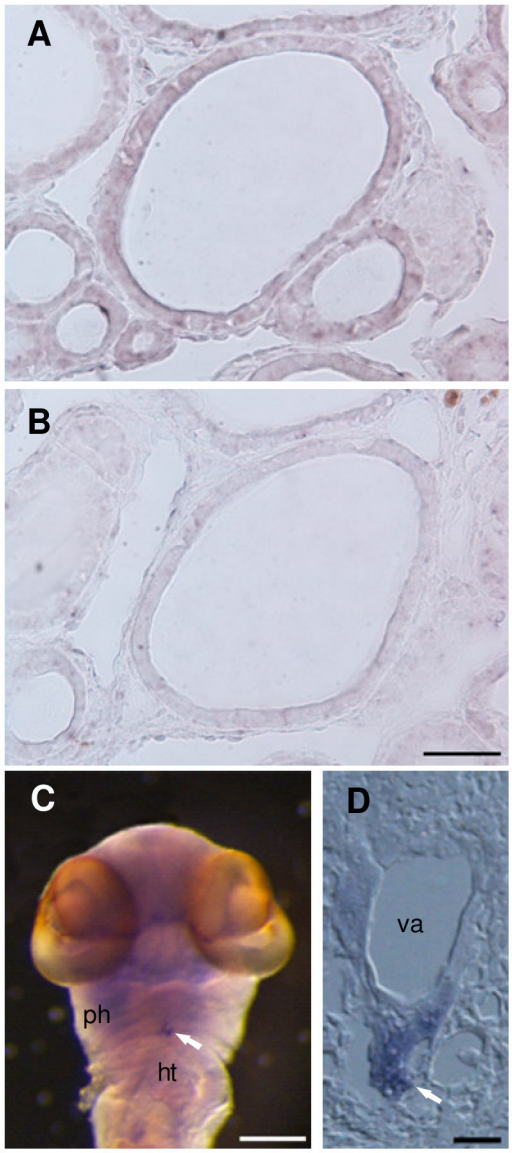
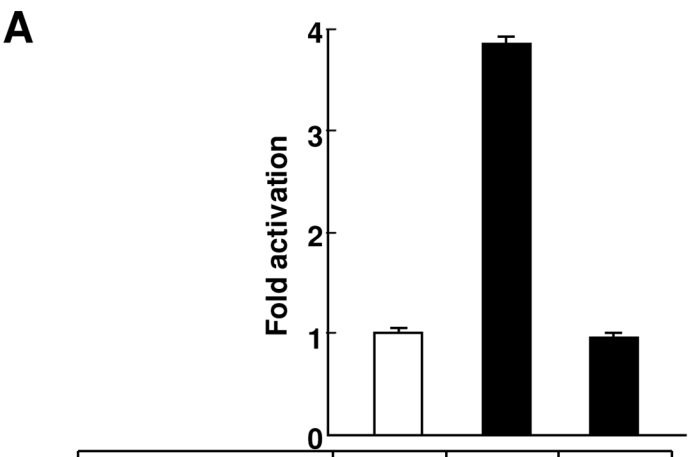
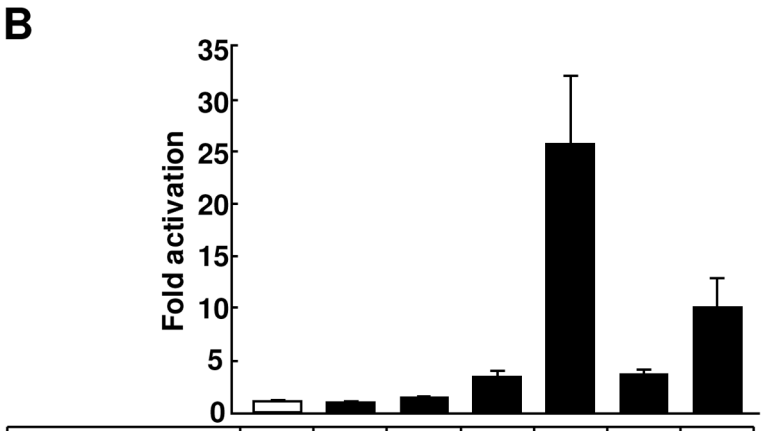


Figure 5.

In situ hybridization histochemistry for Pax8 mRNA in the thyroid gland of the adult trout (A) and in the developing thyroid of the trout embryo (C, D). A: Follicular cells of the thyroid gland showed hybridization signals with the DIG-labelled antisense Pax8 cRNA probe. B: Control section to (A). No signal was seen by hybridization with the sense probe. C: Ventral view of the hybridized 15 dpf embryo showed the signal (arrow) in the pharyngeal region, as a single patch close to the heart tube (ht). The location of the signal appears to correspond to that of the thyroid primordium in the zebrafish embryo (Porazzi et al., 2009). ph, pharynx. D: A transverse section revealed that the signal (arrow) originated from the thyroid primordial cells near the developing ventral aorta (va). The scale bar for A and B = 25 μm; C = 250 μm; D = 20 μm.



Fold activation	1.0	3.9	1.0
pBK-CMV	+	-	-
Rt Pax8a	-	+	-
Rt Pax8b	-	-	+



Fold activtion	1.0	0.9	1.1	3.7	25.0	3.7	10.2
pBK-CMV	+	-	-	-	-	-	-
Rt Pax8a	-	+	-	-	+	-	+
Rt Pax8b	-	-	+	-	-	+	+
Rat Nkx2-1	-	-	-	+	+	+	+

Figure 6.

Transactivation potential of rainbow trout Pax8a and Pax8b for the 713-bp rat TPO gene promoter (A) and for the 6.3-kbp human TPO gene upstream region (B). A: The rat TPO promoter was transactivated by trout Pax8a, but not by Pax8b. B: The trout Pax8a and rat Nkx2-1 showed a remarkable synergy on the transactivation from the human TPO upstream region. On the other hand, trout Pax8b had no synergistic effect with rat Nkx2-1, and inhibited the synergy of Pax8a and Nkx2-1. Values are expressed as fold of activation above

that obtained with the control vector, and represent the average of six independent experiments. Each bar depicts the mean value ± SEM. Rt: Rainbow trout.

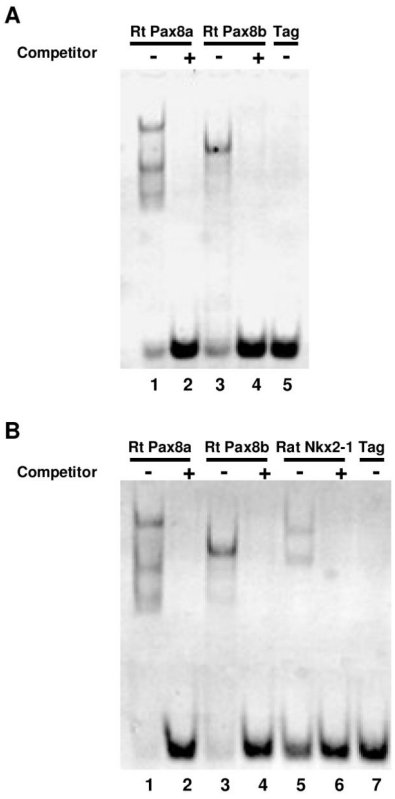


Figure 7.

Binding of rainbow trout Pax8a, Pax8b, and rat Nkx2-1 to oligonucleotide CT (A) or oligo B (B). EMSA was performed with His-tag fused recombinant transcription factor proteins, and FAM-labelled oligonucleotide CT (A) or oligo B (B) as probes. The incubation with rainbow trout Pax8a (lane 1), Pax8b (lane 3), or rat Nkx2-1 (B, lane 5) resulted in shifted bands. The binding specificity was assessed in the absence (−) and presence (+) of a 100-fold molar excess of non-labelled oligonucleotide CT (A) or oligo B (B) as competitors (lanes 2, 4, and 6), and further by using the Tag polypeptide produced from the expression vector pET32a (A, lane 5; B, lane 7). Rt: Rainbow trout.